SUPPLEMENTARY MATERIALS

For

Tobacco Smoke Induces the Generation of Procoagulant Microvesicles from Human Monocyte/Macrophages

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SUPPLEMENTAL METHODS

Reagents and antibodies

PhosphoPlus antibody kits against three major mitogen-activated protein kinases (MAPK), namely, Jun N-terminal kinase (JNK, catalog #9250), p38 MAPK (cat#9210), and extracellular signal-regulated kinase (ERK, cat#9100), were purchased from Cell Signaling Technology (Beverly, MA, USA). Monoclonal antibodies (mAb) against human TF, with or without FITC labeling, were from American Diagnostica (Stamford, CT). Phycoerythrin (PE)-labeled annexin-V was from BD Pharmingen (San Jose, CA). Inhibitors of JNK (SP600125, Cat#5567), p38 (SB202190, Cat#7067), ERK (U0126, Cat#U120), MAP kinase/ERK kinase (MEK, PD98059), Ras (farnesyl transferase inhibitor, FTI) were obtained from Sigma Chemical Company (St. Louis, MO). The caspase 3 inhibitor, Z-DEVD-FMK, was from R&D Systems (Minneapolis, MN).

Preparation of tobacco smoke extract

Research-grade cigarettes were obtained from the Reference Cigarette Program at the University of Kentucky. Tobacco smoke extract (TSE, 100%) was prepared by using a device to bubble mainstream smoke from five cigarettes through 10 ml of serum-free RPMI medium containing 0.2% BSA (RPMI/BSA), at one cigarette per 7-8min, simulating the burning rate of typical smoking.[1-4] The TSE was adjusted to a pH of 7.4 and then sterilized by passage through a filter with a 0.22-µm size cut-off. Our initial studies indicated that a single freeze-thaw cycle did not alter the biologic effects of TSE on cultured cells, and so TSE was aliquoted and stored at -80°C. To ensure consistency among preparations, each batch of TSE was standardized according to its absorbance at 320nm[5, 6] by comparison with aliquots of a standard preparation that was made at the beginning of the study.

Cell culture

The human THP-1 monocytic cell line (ATCC, Manassas, VA) was maintained in suspension culture in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin, as recommended by the ATCC. Primary human monocyte-derived macrophages (hMDMs) were prepared from fresh Buffy coats by selecting monocytes by adherence followed by differentiation into macrophages as described.[7-9] At the beginning of each experiment, THP-1 monocytic cells or hMDMs were transferred to serum-free RPMI/BSA supplemented with different concentrations of TSE, ranging from 0% (control) to 3.75% (v:v), and then incubated at 37ºC for 2-20 h (0h denotes harvest immediately before adding TSE). In time-course studies of kinase activation, cells were placed into serum-free medium simultaneously and then harvested simultaneously; TSE was added at different times before harvest. In experiments using kinase or caspase 3 inhibitors, the compounds were added to cells 1h before the addition of TSE and remained until the end of the study, at concentrations of 100 µM Z-DEVD-FMK, 10 µM SP600125, 10 µM SB202190, 10 µM U0126, 20 µM PD98059, and 20 µM FTI.

Flow cytometric characterization of microvesicles and cells, Total MV quantification by ELISA

Quantifications of MV generation, TF display by MVs and cells, and markers of early and late apoptosis were performed according to our published protocols.[9, 10] In brief, at the end of each incubation, THP-1 cells, which grow in suspension, were fixed in their conditioned medium by the addition of paraformaldehyde to a final concentration of 1%. Conditioned medium from hMDMs was also fixed and then supplemented with latex beads as a reference for MV counts. The number of MVs in each sample was quantified by flow cytometry (FACSCalibur, Becton Dickinson), using gating criteria based on particle size, as detected by forward scatter, and surface exposure of PS, as detected by staining with PE-labeled annexin-V (BD Pharmingen).[9, 10] For THP-1 cell suspensions, results are reported as MVs per 1000 cells. Because hMDMs are adherent, we added 25000 15-µm latex beads (Molecular Probes) to each 100 µL of their conditioned medium as a reference, and our results are reported as absolute numbers of MVs per ml of conditioned culture medium. The portion of these PS-positive MVs that were also TF-positive was quantified by simultaneous staining with a FITC-labeled anti-human TF mAb (Cat# 4508CJ, American Diagnostica)[9].

As a second, independent method, the Zymuphen MP-Activity ELISA kit (Anira, Mason, OH) was used to quantify total PS-positive MVs in culture supernatants that we prepared by low-speed centrifugation to remove cells. The MV ELISA assessment was performed according to the manufacture's instruction by using 20-fold dilutions of THP-1-conditioned media and 5-fold dilutions of hMDM-conditioned media. Results are expressed as PS equivalents (nM PS eq).

Cell-surface display of TF was analyzed by flow cytometry using the same FITC-labeled anti-TF mAb.[9] Cell-surface exposure of PS, an early marker of apoptosis, was detected by staining of cells with PE-labeled annexin-V following by flow cytometric analysis. Late-stage apoptosis was assessed by terminal deoxynucleotidyl transferase FITC-dUTP nick end labeling of the cells (TUNEL; APO-DIRECT kit, BD Pharmingen), also quantified by flow cytometry.

Assessments of tissue factor mRNA and protein expression and tissue factor procoagulant activity
Total RNA from 2 x 10^6 cells was isolated with 1 ml Trizol® reagent (GiBCo BRL, Carlsbad, CA) according to the manufacturer's instructions. Tissue factor mRNA expression was assessed by real-time quantitative reverse-transcriptase PCR (qPCR). Primers and probes for qPCR were synthesized by the Gene Expression Facility at the University of North Carolina (Dr. Hyung Suk Kim, Director; Chapel Hill, NC), using the following sequences:

Human tissue factor mRNA:
5'- gat aaa gga gaa aac tac tgt ttc ag -3' (sense primer)
5'- tac cgg gct gtc tgt act ct -3' (antisense primer)
F-5'- tca agc agt gat tcc ctc ccg aac ag -3'-Q (probe, where F and Q denote the positions of the fluorophore and quencher)

Human ß-actin mRNA:
5'- ggt cat cac cat tgg caa tg -3' (sense primer)
5'- tag ttt cgt gga tgc cac ag -3' (antisense primer)
F-5'- cag cct tcc ttc ctg ggc atg ga -3'-Q (probe)

Tissue factor procoagulant activity (PCA) was measured with fresh (unfixed, unfrozen) samples using a chromogenic assay for the activation of clotting factor X (active factor Xa; Actichrome TF activity kit, American Diagnostica). In brief, cell-culture supernatants were obtained by low-speed centrifugation of conditioned medium from THP-1 cells or hMDMs after treatment without or with TSE. MVs from 4ml of each supernatant were purified by high-speed centrifugation (100,000 x *g* at 4ºC for 1h), then washed, re-isolated, and re-suspended in 0.2ml of 50 mM Tris-HCl and 100 mM NaCl. The PCA of these purified MVs was analyzed according to the manufacturer's instructions, except that to avoid saturation of the assay, we measured the absorbance at 405 nm of the reaction solutions in a 96-well plate every minute for 60 min with a BioTek Powerwave XS spectrophotometer. The linear portion of the curve was used to calculate the PCA.

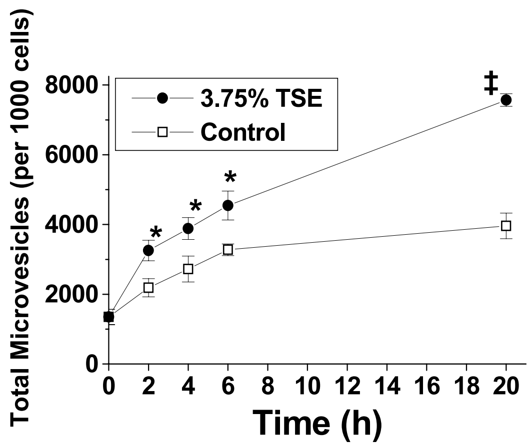
Immunoblots

Cells were extracted into a lysis buffer containing 20 mM Tris/HCl, Ph 7.4, 150 mM NaCl, 1mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 2.5mM sodium pyrophosphate, 1mM Na_3VO_4, and a commercial protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Germany) for 30 min on ice. Samples containing the same amount of total protein were electrophoresed through a 10% SDS-polyacrylamide gel, followed by transfer to a nitrocellulose membrane (Bio-Rad). The membranes were then blocked with skim milk (5%, w:v) and probed with primary antibodies against human JNK, p38, ERK, and their phosphorylated (active) forms, or against TF or ß-actin. Thereafter, detection was accomplished with horseradish peroxidase-conjugated secondary antibodies, to generate a chemiluminescent product (Amersham^{TM} ECL^{TM} Western blot analysis system, GE Healthcare, USA). Signals were quantified using Image J densitometry software and normalized in each independent experiment to values from control (0% TSE) cells.

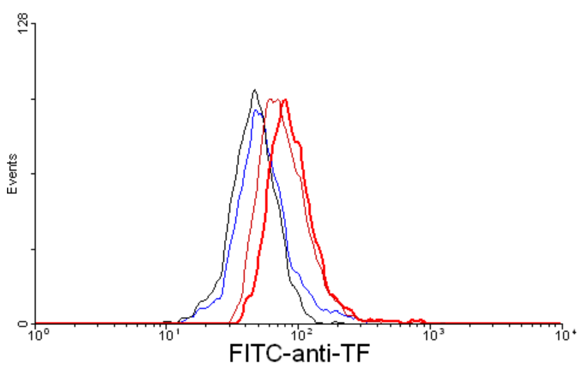
Statistical analyses

Normally distributed data are shown as means ± SEM (n = 4-6). Comparisons amongst three or more groups were performed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) test, with $p<0.05$ considered significant. Comparisons between two groups used the Student's unpaired, two-tailed *t*-test. Comparisons between two groups of non-normally distributed data used the Mann-Whitney rank-sum test.

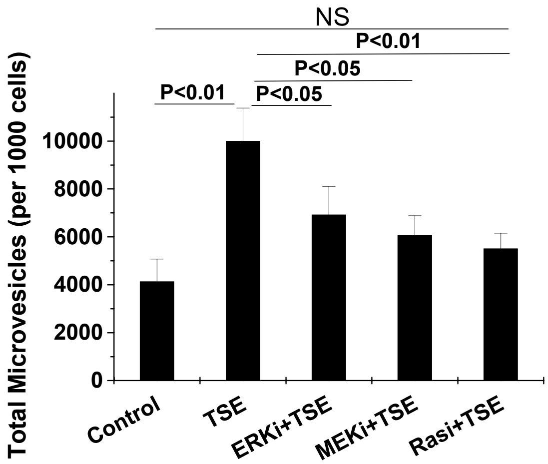
SUPPLEMENTAL FIGURES



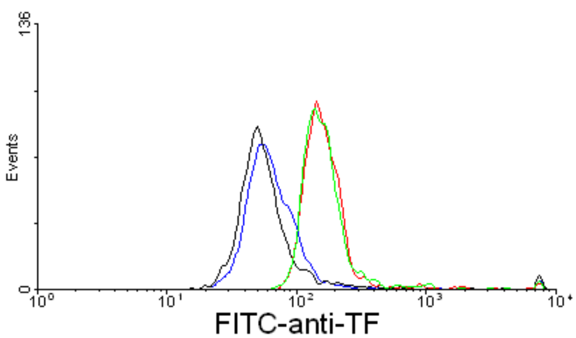
Supplemental Figure I. Exposure of human THP-1 monocytes to TSE increases total MV generation in a time-dependent manner. THP-1 cells were treated for 0 to 20h without (control) or with 3.75% TSE, as indicated, and then samples were analyzed by flow cytometry. *$p < 0.05$, ‡$p < 0.01$ compared by Student's t-test to control values.



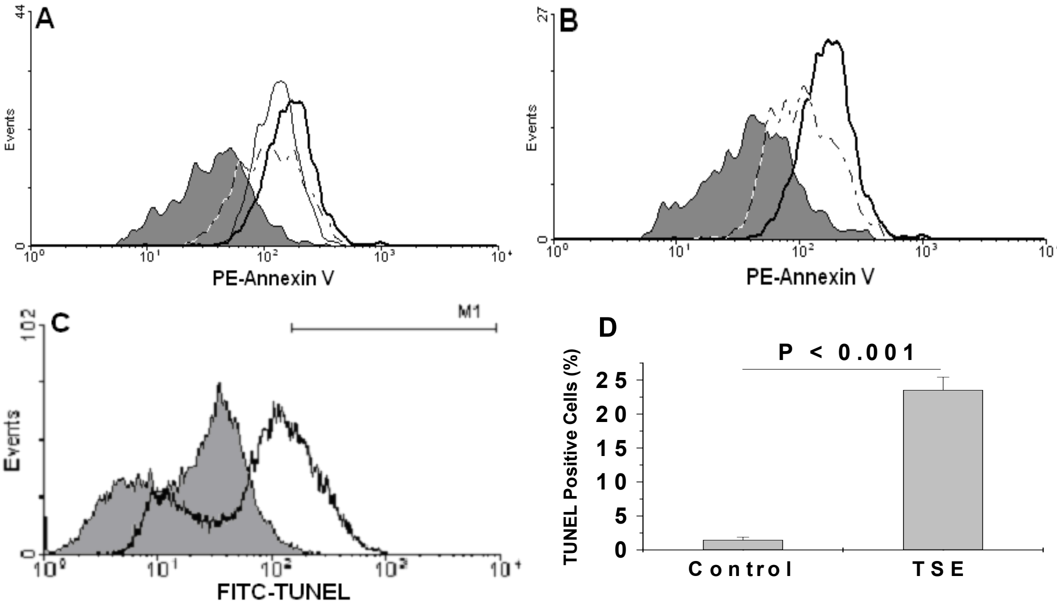
Supplemental Figure II. The figure shows flow cytometric histograms of cell-surface TF display by THP-1 cells after 0h (**black curve**), 6h (**red curve**) or 20h (**orange curve**) of incubation with 3.75% TSE. THP-1 cells treated with 3.75% TSE for 6h but stained with an isotype control antibody are shown by the **blue curve**.



Supplemental Figure III. Treatment of THP-1 monocytes with inhibitors of ERK (U0126, indicated as *ERKi*) or the upstream molecules MEK (PD98059, indicated as *MEKi*) or Ras (FTI, indicated as *Rasi*) essentially blocked the ability of TSE (3.75%) to induce the generation of total MVs, as measured by flow cytometry. P< 0.001 by ANOVA; individual pairwise comparisons were performed by SNK.



Supplemental Figure IV. No effect of ERK inhibition on TSE-induced display of TF on the surface of THP-1 monocytes. The **black curve** shows a representative histogram from flow cytometry of TF display on the surface of control THP-1 cells incubated for 6h without TSE. The **blue curve** shows the surface TF staining of THP-1 cells after 6h treatment with ERK inhbitor U0126. The **red curve** shows surface TF staining of THP-1 cells after a 6h treatment with 3.75% TSE. The **green curve** shows surface TF staining of THP-1 cells treated with both the ERK inhbitor U0126 and 3.75% TSE.



Supplemental Figure V. TSE treatment induces cell-surface PS exposure and nuclear TUNEL staining. Panel A (dose-response) shows representative histograms from flow cytometry of Annexin V staining after 20-h incubations of THP-1 cells with 0 (filled grey), 1.25% (dash line), 2.5% (light line), and 3.75% (heavy line) TSE. Panel B (time course) shows histograms of Annexin V staining after 0h (filled grey), 6h (dash line), or 20h (heavy line) incubations of THP-1 cells with 3.75% TSE. Panel C shows representative histograms of TUNEL staining of hMDMs after 20-h exposure to 0% TSE (*filled grey curve*) or 3.75% TSE (*black line*). The horizontal line (*M1*) indicates intensities considered to be TUNEL-positive for quantification in Panel D. Panel D indicates quantification of TUNEL-positive hMDMs after exposure to 0% (*Control*) or 3.75% TSE (*TSE*). The P-value was computed using Student's t-test.

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